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A model for cGMP signal transduction in *Dictyostelium* in perspective of 25 years of cGMP research

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Abstract

The chemoattactant mediated cGMP response of *Dictyostelium* cells was discovered about twenty-five years ago. Shortly thereafter, guanylyl cyclases, cGMP-phosphodiesterases and cGMP-binding proteins were detected already in lysates, but the encoding genes were discovered only very recently. The deduced proteins appear to be very different from proteins with the same function in metazoa. In this review we discuss these new findings in perspective of the previously obtained biochemical and functional data on cGMP in *Dictyostelium*.

Introduction

Vegetative *Dictyostelium discoideum* cells can respond chemotactically to a number of chemicals that are secreted by bacteria, their natural food source. Among these compounds is folic acid, the most studied chemoattractant in growing *Dictyostelium discoideum* cells. When bacteria are scarce, cells become sensitive to cAMP that serves as the first messenger in cell aggregation. About 25 years ago, it was discovered that cGMP is produced intracellularly when cells are exposed to chemoattractants; the presence of cGMP in *Dictyostelium* had already been suggested 2 years earlier (Putnam and Pedersen, 1975). Probably the first experiment was performed with an pterin-like compound purified from yeast extract that specifically attracts *Dictyostelium lacteum*. This experiment was designed to investigate the activation of adenylyl cyclase in a species that does not use cAMP as chemoattractant. No changes in cAMP levels were observed upon stimulation with the attractant, but instead a rapid and large increase of intracellular cGMP was detected in the same extracts (Mato and Konijn, 1977). Subsequent experiments with folic acid or cAMP stimulation of *Dictyostelium discoideum* cells resulted in a similar cGMP response (Mato *et al.*, 1977a; Wurster *et al.*, 1977). Shortly hereafter, cGMP-degrading and cGMP-producing activities were found in *Dictyostelium* lysates (Mato *et al.*, 1977b; Ward and Brenner, 1977), as well as cGMP-binding activity (Mato *et al.*, 1978). In the 1980s several mutants were isolated displaying aberrant cGMP metabolism as well as altered chemotaxis, suggesting that cGMP plays a role in chemotaxis. Recently we identified the genes encoding guanylyl cyclases, cGMP-phosphodiesterases and cGMP-binding proteins (Roelofs *et al.*, 2001b,c; Bosgraaf *et al.*, 2002a; Goldberg *et al.*, 2002), that probably constitute the main components of the cGMP signal transduction pathway. The encoding proteins appear to be very different from proteins with the same function in metazoa, and therefore their biochemistry is not always easily interpreted. The aim of this review is to discuss the previously obtained biochemical and functional data on cGMP in *Dictyostelium* in the perspective of the recently identified genes.

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Guanylyl cyclases

Guanylyl cyclases (GCs) are enzymes that catalyze the formation of cGMP from GTP (Figure 1) and are found in a wide variety of organisms, including protozoa, vertebrates, and cyanobacteria. The first experiments in *Dictyostelium* showed that the GC activity can be activated considerably by chemoattractants and is also enhanced by ATP and non-hydrolyzable GTP analogues (Mato and Malchow, 1978; Mato, 1979; Janssens and de Jong, 1988; Janssens *et al.*, 1989). Initially, the activity was found to be Mn^{2+} dependent and almost exclusively cytosolic (Padh and Brenner, 1984). Later it was discovered that activity with the more physiological cofactor Mg^{2+} is restricted to the membrane and under these conditions ATP inhibits the enzyme (Janssens *et al.*, 1987). The enzyme, which is inhibited by Ca^{2+} ions (Janssens and de Jong, 1988; Valkema and Van Haastert, 1992), was found to be partially or loosely attached to the membrane (Mato and Malchow, 1978; Padh and Brenner, 1984; Janssens *et al.*, 1987).

The cloning of the Dictyostelium *cyclases*

In the *Dictyostelium* genome, eight cyclase domains can be recognized, four of which encode adenylyl cyclase (AC) domains. The aggregation-specific ACA is a classical AC containing 12 transmembrane regions and two cyclase domains. On the other hand, the germination

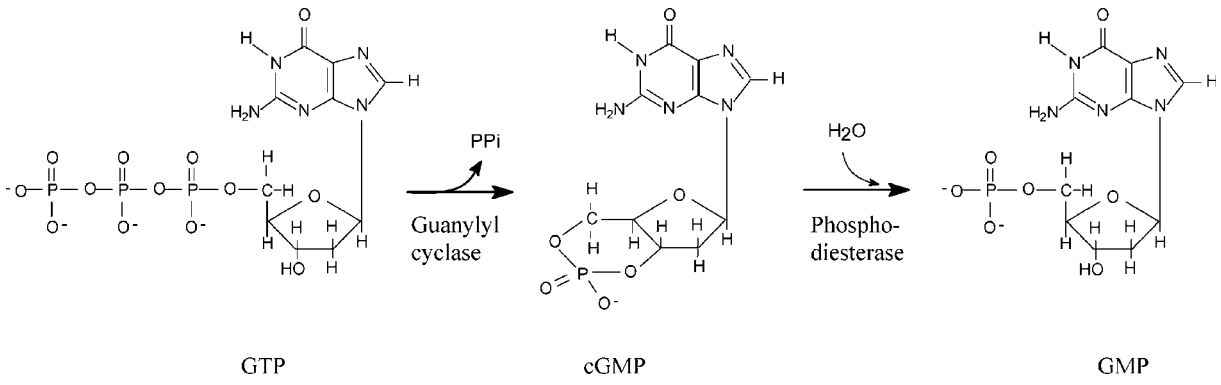


Fig. 1. Structure of cGMP, its precursor GTP and its breakdown product GMP.

specific ACG has the topology of a GC containing one cyclase domain and one membrane spanning region. The developmental AcrA has seven predicted transmembrane spanning regions and one cyclase domain. The other four cyclase domains are GCs that are encoded by two recently cloned genes (Roelofs *et al.*, 2001b,c). A double knock-out, in which both genes were inactivated, had no detectable cGMP anymore, suggesting these are the only two GCs in *Dictyostelium discoideum* (Roelofs and Van Haastert, 2002). Interestingly, both proteins have the topology of ACs. In soluble GC (sGC) the two cyclase domains are in the same molecule, whereas classical metazoan soluble GCs are composed of two proteins that form a heterodimer. *Dictyostelium* guanylyl cyclase A (GCA) has 12 membrane spanning regions, whereas conventional membrane-bound GCs have only one transmembrane region. The topology of GCA has also been observed in other protozoan GCs (Linder and Schultz, 2002).

Properties of the guanylyl cyclases

Although the proteins are very different in topology, the biochemical properties of GCA and sGC display many similarities (Roelofs and Van Haastert, 2002): Both enzymes are activated *in vivo* by folic acid and cAMP and addition of GTPγS *in vitro* leads to similar stimulation of the enzymes (1.5-fold increase of V_{max} and 3.5-fold increase of K_m for GTP). Furthermore, both enzymes are inhibited by Ca^{2+} ions though the inhibition constants are somewhat different (50 nM for GCA and 200 nM for sGC). Disruption of the *sgc* gene reduces GC activity to about 30% in vegetative cells and to about 10% in aggregation-competent cells. Another difference between the two enzymes is the relative activity in the presence of bivalent cations: sGC is 5-fold more active with 2 mM Mn^{2+} than with 2 mM Mg^{2+}, whereas GCA is 3-fold less active with Mn^{2+} than with Mg^{2+}. For sGC the Mn^{2+}- and Mg^{2+}-dependent activities represent different subpopulations of the enzyme (Roelofs *et al.*, 2001b). With Mn^{2+} most of sGC activity is soluble and is affected by neither GTPγS nor calcium ions, while with the more physiological Mg^{2+}, the activity is restricted to the particulate fraction and is strongly stimulated by GTPγS and inhibited by Ca^{2+}. Experiments in which particulate and cytosolic fractions of different mutants where mixed indicated that calcium inhibits membrane association of sGC, thus blocking its Mg^{2+}-dependent activity (J. Roelofs and P.J.M. Van Haastert, unpublished results).

Both sGC and GCA are affected similarly by GTPγS (Roelofs and Van Haastert, 2002), which by itself is a poor substrate for total GC activity (Schulkes *et al.*, 1992). This suggests that both enzymes are activated via a similar mechanism, which involves a GTP-binding protein. *In vivo*, stimulation of GC activity is completely dependent on the heterotrimeric G-protein Gα2βγ (Okaichi *et al.*, 1992; Carrel *et al.*, 1994; Wu *et al.*, 1995; Root *et al.*, 1999). However, several indications suggest that GTPγS-mediated activation of sGC and GCA *in vitro* is not due to the activation of a heterotrimeric G-protein. For example, in cells lacking the only known Gβ subunit, GC activity can still be activated by GTPγS (Wu *et al.*, 1995). Further evidence comes from a study in which GCA was converted to an AC by introducing three point mutations (Roelofs *et al.*, 2001). Remarkably, the protein retained its GTPγS dependence, also when expressed in Gβ-null or Gα2/4 double null cells. The properties of GTPγS-stimulation are consistent with a mechanism that involves a small GTP-binding protein of the Ras superfamily. This is supported by the observation that disruption of the gene encoding the putative Ras exchange factor AleA or the gene coding for the Ras interacting protein RIP3 led to a marked reduction of the cAMP-induced cGMP response (Lee *et al.*, 1999). Furthermore, the small G-protein Rap1 was found to be involved in osmotic shock induced cGMP formation, since introduction of antisense RNA inhibited this response (Kang *et al.*, 2002). A model for the regulation of GCA and sGC is presented in Figure 2.

Phosphodiesterase

The activation of GC upon receptor stimulation is transient and is inhibited with a $t_{1/2}$ of about 15 s (Schoen *et al.*, 1996). Because the cGMP is rapidly

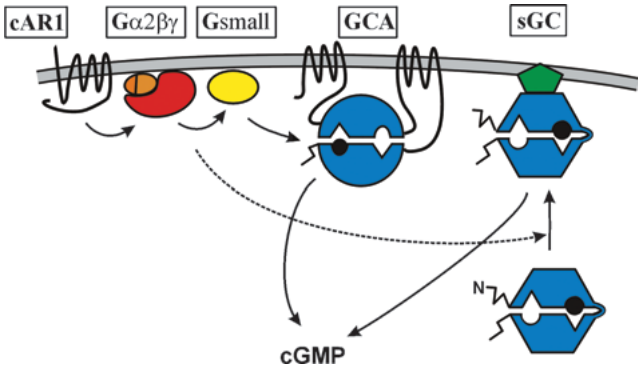
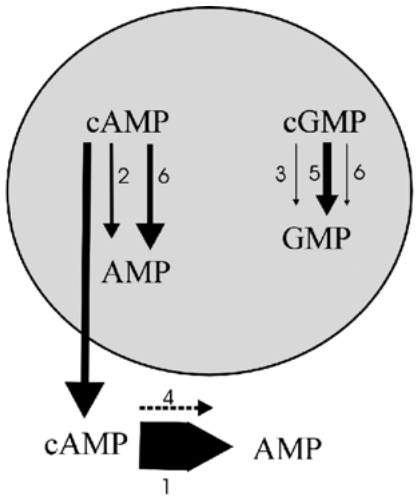


Fig. 2. Schematic overview of the activation of *Dictyostelium* guanylyl cyclases. Upon cAMP stimulation, the cAMP receptor (cAR1) transmits the signal via the heterotrimeric G-protein Gα2βγ to a small G-protein (Gsmall), which in turn activates the membrane-bound GCA and the soluble sGC. The sGC binds to the membrane *via* an unknown factor shown in green.

degraded inside the cell, this leads to a short burst of cGMP. The degradation of cGMP to GMP is accomplished by specific enzymes called phosphodiesterases (PDEs) (Figure 1). In *Dictyostelium*, a cGMP-specific PDE activity was observed that can be separated from cAMP-PDE activity (Dicou and Brachet, 1980). Already the first report on cGMP-PDE activity reported that the enzyme activity displays positive cooperativity (Mato *et al*., 1977b). Subsequent experiments using a number of cGMP-derivatives indicated that hydrolysis of cGMP and activation of the enzyme by cGMP occur at different sites of the protein (Van Haastert *et al*., 1982; Kesbeke *et al*., 1985). The enzyme can be activated about 4-fold by cGMP or 8-Br-cGMP, which is a poorly hydrolysed cGMP-analogue that preferentially binds to the activator site (Van Haastert and Van Lookeren Campagne, 1984). The identification of chemically mutated cells that lack all or almost all cGMP-stimulated cGMP-PDE activity has provided a useful tool to study the cGMP-regulated cGMP-PDE in *Dictyostelium* (Ross and Newell, 1981; Van Haastert *et al*., 1982). These mutants are referred to as 'Streamer F' since they form abnormally large aggregation streams when plated on bacteria. Due to the strongly reduced cGMP-PDE activity, these cells exhibit a four times prolonged and 2-fold augmented cGMP response.

Cloning of the phosphodiesterases

In *Dictyostelium* six PDE encoding genes have been cloned thus far; we propose to address the proteins as PDE1-6 in the order of their cloning (see Figure 3). PDE1 is a dual specificity enzyme encoded by the *pdsA* gene and has an important role in degrading extracellular cAMP (Podgorski *et al*., 1988). The intracellular cAMP is degraded predominantly by the *regA* gene product (PDE2), which not only bears a cAMP-specific PDE domain, but also an N-terminal receiver domain that forms a functional phospho-relay system with the rdeA phospho-transfer protein (Chang *et al*., 1998;



Proposed name	Original gene name	Acronym	Characteristic
PDE1	*pdsA*		Dual specificity extracellular PDE
PDE2	*regA*		cAMP-specific PDE with response regulator
PDE3	*pde3*		cGMP-specific PDE
PDE4	*pde4*		Transmembrane putative PDE
PDE5	*gbpA*	PDED	cGMP-activated cGMP-PDE
PDE6	*gbpB*	PDEE	cAMP/cGMP-activated cAMP/cGMP-PDE

Fig. 3. Schematic overview of the relative activity of the six identified *Dictyostelium* phosphodiesterases. *Dictyostelium* cells have three functional pools of cyclic nucleotides: extracellular cAMP as chemoattractant, intracellular cAMP as inducer of gene expression and intracellular cGMP as second messenger for chemotaxis. The relative contribution of the concerned PDE to the total cAMP- or cGMP-degrading activity of each pool is indicated by the thickness of the arrows. The numbers of the PDEs are defined in the table below, which also contains the original names of the genes, acronyms, and the main characteristics of the enzymes.

Shaulsky *et al*., 1998; Thomason *et al*., 1998, 1999). Based on sequence data, PDE4 is a putative cAMP-PDE that has two transmembrane segments and an extracellular catalytic domain; the gene is expressed predominantly in the later stages of development (S. Bader *et al*., unpublished observations).

Apart from the non-specific extracellular PDE1, no cGMP-degrading PDEs had been cloned until 2 years ago. The contribution of PDE1 to intracellular cGMP degradation is low since this enzyme is found mainly on the cell surface and in the extracellular space. However, since cell lysates contain considerable amounts of this very active enzyme, probably on its way to secretion, PDE1 masks cGMP-specific PDE activity unless the enzyme is inhibited by dithiotreitol. Recently, the sequencing of the *Dictyostelium discoideum* genome has proven fruitful, since three cGMP-PDEs have been identified in the past 2 years.

Firstly, a cGMP-specific PDE termed DdPDE3 was identified that contains a catalytic site which shows high sequence homology with mammalian PDEs (Kuwayama *et al*., 2001). Apart from a 300 amino acid catalytic domain the predicted protein only contains a N-terminal Asp- and Gln-rich domain with no clues for a regulatory

function. The other two cGMP-PDEs were unexpectedly discovered in a search for cGMP targets and were termed cGMP-binding protein A and B (GbpA and GbpB) or PDE5 and PDE6 respectively because they were the fifth and sixth PDEs that were cloned in *Dictyostelium* (see table in Figure 3) (Bosgraaf *et al*., 2002a,b; Goldberg *et al*., 2002). Both enzymes contain two tandem C-terminal cyclic nucleotide binding domains (cNBD) that probably serve as allosteric cGMP-binding sites for a metallo β-lactamase domain that comprises the catalytic center (Meima *et al*., 2002). The domain topology of PDE5 and PDE6 is very unusual: in all other identified cyclic nucleotide activated PDEs the allosteric sites are formed by GAF domains (Aravind and Ponting, 1997; Conti and Jin, 1999) that are structurally entirely unrelated to cNBDs, and cGMP is hydrolyzed by a type I PDE domain that is unrelated to the β-lactamase domain.

Properties of the phosphodiesterases

The DdPDE3 protein was analyzed by overexpression in *Dictyostelium* and *E. coli* (Kuwayama *et al*., 2001), which revealed that the enzyme has a high affinity for cGMP (0.22 μM), high selectivity for cGMP over cAMP (670:1) and a low V_{max} (2 pmol/min/mg). The relative contribution of DdPDE3 to cGMP degradation *in vivo* is highest when the cGMP concentration is low because the activity of DdPDE3 cannot be stimulated by cGMP or 8-Br-cGMP and the enzyme is easily saturated due to its low V_{max} and K_m values. Thus the main function of this enzyme seems to be the maintenance of low basal cGMP levels.

Cell lines in which the gene coding for PDE5 has been disrupted have lost all cGMP-activated cGMP-PDE activity and show a cAMP-induced cGMP response reminiscent of that of *StmF* mutants (Bosgraaf *et al*., 2002b; Meima *et al*., 2002). The biochemical properties of PDE5 as examined in knock-out and overexpression studies are identical to that of the *StmF* enzyme (K_m 5.2 and 20 μM for unstimulated and stimulated activity respectively, V_{max} 390 pmol/min/mg, K_A 160 nM), strongly suggesting that PDE5 is not active in these Streamer mutants. However, we have not been able to identify the primary defect in *StmF* strain NP368 as no mutations in the gene coding for PDE5 were found that could explain the defective PDE activity. *StmF* strain NP377 shows reduced PDE5 mRNA levels, but also no mutation in the gene causing this effect was found (Meima *et al*., 2002).

Overexpression of PDE6 in a *pde5*$^-$/*pde6*$^-$ cell line showed that PDE6 is a dual specificity enzyme with 9-fold higher activity towards cAMP than towards cGMP (K_m 200 μM for cAMP and 800 μM for cGMP; V_{max} 5200 pmol/min/mg for cAMP and 2400 pmol/min/mg for cGMP) (Bosgraaf *et al*., 2002b). Interestingly, the enzyme can be activated by both cGMP and cAMP, the latter being the most potent (K_A 2.3 and 0.7 μM respectively). This observation indicates that cAMP- and cGMP-levels can influence each other via PDE6. Mutants with a deletion of the gene coding for PDE6 show a nearly normal cGMP response, but an enhanced cAMP response, in agreement with cAMP being the better substrate of the enzyme. The notion that intracellular cAMP is the main physiological substrate of PDE6 is also suggested by the observation that *pde6* null cells are sporogenous, as are mutants that lack the cAMP-specific enzyme RegA (Shaulsky *et al*., 1998).

PDE5 comprises the main intracellular cGMP hydrolyzing activity in *Dictyostelium* and it can be calculated that at cGMP peak concentrations (1 μM), it makes up about 78% of the total cGMP-degrading activity (Bosgraaf *et al*., 2002b). Although disruption of PDE6 in a wild-type background does not lead to an enhanced cGMP response, disruption in a *pde5*-null cell line leads to a dramatically enhanced cAMP-induced cGMP response. This very large cGMP response in *pde5*/*pde6* double null cells is completely wiped out upon 50-fold overexpression of PDE6. These observations indicate that PDE6 does hydrolyze cGMP, but is much less active than PDE5. We estimate that PDE6 contributes only about 5% to the total cGMP-degrading activity at cGMP peak concentrations and the main function of this enzyme is probably cAMP degradation in multicellular development. Summarizing, six PDEs have been identified in *Dictyostelium*, four of which can degrade cGMP. The relative contribution of each of these enzymes to the total cyclic nucleotide degrading activity is indicated by the thickness of the arrows in Figure 3.

cGMP-binding activity

Shortly after cGMP was found to be present in *Dictyostelium discoideum*, cGMP-binding activity was also demonstrated (Mato *et al*., 1978; Rahmsdorf and Gerisch, 1978). This activity, which was found to be mainly soluble, appeared to be very labile, which might account for the rather diverse characteristics that have been reported over time. The cGMP binding was unaffected by EGTA, $CaCl_2$, ATP, 5′-AMP or 5′-GMP, whereas cAMP was found to be inhibitory at >1000 times higher concentrations (see also Van Haastert *et al*., 1982). In one study a single cGMP-binding fraction eluted from a DEAE-cellulose column with an apparent mass of several hundred thousand dalton (Rahmsdorf and Gerisch, 1978). In another study, three peaks containing cGMP-binding activity were separated by gel filtration chromatography, of which the largest had an apparent mass of 250 kDa (Mato *et al*., 1979). Several other studies also described multiple fractions that display cGMP-binding capacity, but these were proposed to be degradation products of a larger protein (Parissenti and Coukell, 1989). The number of cGMP-binding sites is a few thousand per cell with a K_d between 0.5 and 5 nM (Mato *et al*., 1978, 1979; Van Haastert *et al*., 1982; Parissenti and Coukell, 1986).

Whereas in other organisms the cGMP-activated protein kinase PKG is a major target of cGMP, no protein kinase activity was associated with the partly purified cGMP-binding activity from *Dictyostelium* when using histone 2a, histone type VII or Kemptide as a substrate (Rahmsdorf and Gerisch, 1978; Mato *et al*., 1979; Parissenti and Coukell, 1986). However, in a separate study a protein kinase was purified with an antibody, which was found to be activated 3-fold by cGMP or cAMP with half-maximal activation at 1 and 20 nM respectively (Wanner and Wurster, 1990). The labile kinase preparation phosphorylated histone 2b but not Kemptide and histone 2a, and contained several components with a molecular weight range of 20–130 kDa. Unfortunately, the cGMP-binding activity and other biochemical characteristics were not further determined, leaving open the question whether this enzyme also comprises the main cGMP-binding activity in *Dictyostelium*.

Parissenti and Coukell (1989) reported the isolation and stabilization of a cGMP-binding activity with an apparent mass of 160 kDa. This activity could exist in two interconvertable forms, one with fast dissociation of cGMP ($t_{1/2} < 1$ min) and a K_d of about 6 nM (F-type), and one with a much slower dissociation rate ($t_{1/2}$ of 68 min) and a K_d of 2 nM (S-type). Conversion of F-type to S-type cGMP-binding activity is evoked by adding DNA, in particular by octamers of deoxyguanosine or deoxycytosine (Parissenti and Coukell, 1990). Moreover, about half of the S-type cGMP-binding activity was found to be associated with nuclei, suggesting an unexpected nuclear function of the cGMP-binding protein (Butler and Coukell, 1992). Furthermore, in filipin permeabilized cells, the cGMP-binding activity was found to have a $t_{1/2}$ for dissociation of cGMP of about 70 min, indicating that the cGMP-binding activity might exist in its S-type *in vivo*. However, this observation contradicts a study in which the cGMP-binding capacity at different time points after cAMP stimulation was measured, which indicated a dissociation of cGMP from the cGMP-binding activity *in vivo* with a half-life of about 2 min, which corresponds better to the F-type cGMP-binding activity (Van Haastert *et al*., 1982).

In wild-type cells, the partially purified cGMP-binding activity activates the particulate GC activity about 2-fold and the addition of DNA enhances this effect (Kuwayama and Van Haastert, 1996). Furthermore, the same cGMP-binding fraction inhibits GC activity when ATP is added, an effect that is enhanced by the addition of 50 nM 8-Br-cGMP. These findings suggest that the cGMP-binding protein from *Dictyostelium* regulates the Mg^{2+}-dependent GC activity in a complex way. The identification of mutants that have an aberrant cGMP-binding activity has provided some interesting information about this regulating activity (Kuwayama *et al*., 1995; Kuwayama and Van Haastert, 1996). Two mutants, KI4 and KI5, have a cGMP-binding activity with lowered K_d and slow dissociation kinetics. Furthermore, DNA does not alter the dissociation rate, so it seems the cGMP-binding activity is locked in the S-form in these mutants. Interestingly, the partially purified cGMP-binding fraction from these mutants does not activate wild-type particulate GC, suggesting that the cGMP-binding protein is locked in an inhibiting form.

Cloning of the cGMP-binding proteins

Although *Dictyostelium* lysates contain a highly specific, high affinity cGMP-binding activity, PCR reactions using redundant primers for cGMP-binding domains have not given any positive result (H. Kuwayama and P.J.M. Van Haastert, unpublished results). Again the sequencing of the *Dictyostelium discoideum* genome has proven vital to identify genes that do not exactly match sequence expectations, since the genes of four putative non-classical cGMP targets have been identified recently (Goldberg *et al*., 2002). These proteins each possess two tandem cyclic nucleotide binding domains and were termed Gbp for cGMP-binding protein; the two phosphodiesterases PDE5 and PDE6 were already discussed above. In other identified cNBDs, including those from the *Dictyostelium discoideum* PKA regulatory subunit, a RXA motif determines the specificity of the cyclic nucleotide that is bound (Shabb and Corbin, 1992). Interestingly, this motif is absent in the Gbps (except for the first nucleotide binding domain of PDE5), and the homology with the cNBDs of the Gbps is low in this region, suggesting that the molecular basis of cGMP binding to Gbps in *Dictyostelium* is different from cGMP/cAMP binding to the cNBDs of other organisms. In this respect it is interesting to mention that there is substantial homology in other parts of the cNBDs between the Gbps. The identified putative cGMP targets display a unique topology. The N-terminal half of GbpC contains five leucine-rich repeats, a Ras domain and a protein kinase domain that most closely resembles a MAPKKK. The C-terminal half consists of a RasGEF domain and two cNBDs that are separated by a GRAM domain, which is a small domain that probably plays a role in protein–protein interactions (Doerks *et al*., 2000). The GbpD protein is homologous to the C-terminal half of GbpC and also contains a RasGEF domain, a GRAM domain and two cNBDs.

The Ras, RasGEF and MAPKKK domains in GbpC are homologous to different proteins in the metazoan Ras signaling cascade, in which a RasGEF (Sos) activates Ras that stimulates a MAPKKK (Raf). This allows an interesting model in which cGMP binding to one of the cNBDs stimulates an intramolecular cascade leading to the activation of MAPKKK. The RasGEF domain of GbpD may also participate in this reaction scheme (Figure 4). GbpC could easily function as a molecular integrator if other RasGEF or Ras proteins, that are stimulated by other signals, interact with the domains in GbpC. A model of the main cGMP signaling components that have now been identified is represented in Figure 5.

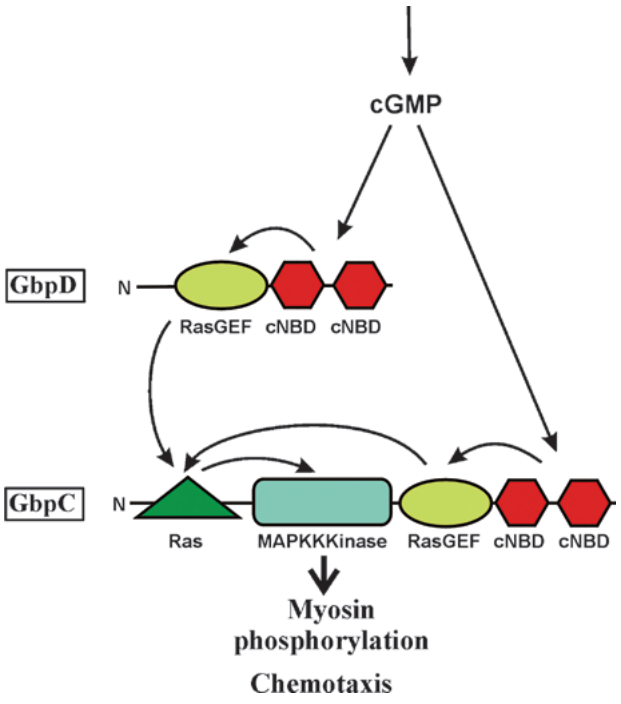


Fig. 4. Model of the possible functioning of GbpC and GbpD. The cyclic nucleotide binding domains (cNBD) of the putative cGMP-binding proteins GbpC and GbpD bind cGMP leading to the activation of the Ras guanine exchange factor domains (RasGEF), which activates the small G-protein domain (Ras) of GbpC. This results in the activation of the kinase domain of GbpC (MAPKKK), which stimulates a phosphorylation cascade eventually leading to the phosphorylation of myosin II and chemotaxis.

Properties of the cGMP-binding proteins

Cell lines in which the GbpC has been disrupted have lost all high affinity ($K_d \sim 4$ nM) cGMP-binding capacity (Bosgraaf *et al.*, 2002a; Goldberg *et al.*, 2002). The remaining cGMP-binding capacity has a much lower affinity ($K_d \sim 500$ nM) and is probably caused by a number of proteins including the allosteric and catalytic sites of PDE5 and PDE6, the regulatory subunit of PKA and possibly GbpD. However, cGMP binding in a *gbpC*$^-$/*gbpD*$^-$ cell is indistinguishable from cGMP-binding in the *gbpC*$^-$ knock out, which might indicate that GbpD does not strongly bind cGMP.

The presence of a protein kinase domain in the GbpC protein raises the question whether it is the previously identified cGMP-activated kinase in *Dictyostelium*. The mass of GbpC (294 kDa) is considerably larger than that of the components in the cGMP-activated kinase preparation (20–130 kDa), but an imaginary proteolytic product that contains the kinase domain and the first cNBD would weigh only 140 kDa. The cGMP-activated kinase is activated half-maximally at 1 nM cGMP (Wanner and Wurster, 1990) and GbpC is the only protein in *Dictyostelium* that binds cGMP effectively at this low concentration. The cGMP-regulated kinase activity was found to be activated by cAMP as well, albeit at 20-fold higher concentrations than cGMP, whereas GbpC binds cAMP with more than 1000-fold lower affinity than cGMP. Since we cannot exclude that one of the cNBDs of GbpC binds cGMP with high affinity and selectivity (which is detected in the intact protein), while the other binds cGMP with lower affinity and selectivity (which would be present in the truncated

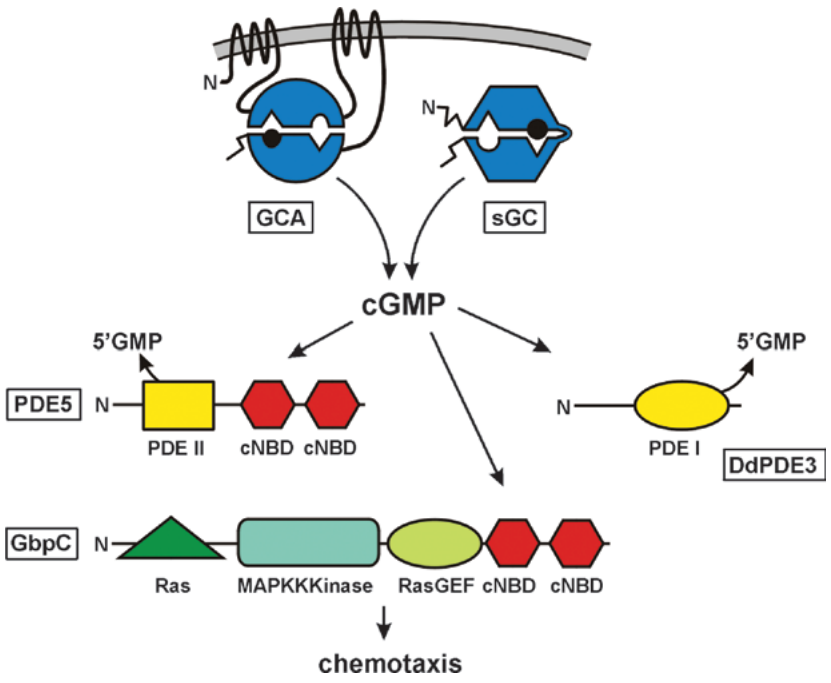


Fig. 5. Proposed model for the function of the main cGMP signaling molecules. cGMP is produced by the guanylyl cyclase GCA and sGC, leading to the activation of the cGMP-binding protein GbpC and chemotaxis. cGMP can be degraded by the cGMP-stimulated PDE5 protein which is distantly related to class II PDEs, and by DdPDE3, a non-stimulated class I PDE.

protein), it cannot be excluded that the previously identified cGMP-regulated protein kinase was part of GbpC.

The function of cGMP in *Dictyostelium*

Since cGMP is produced in response to chemotactic stimulation, it has long been thought that it plays a role in directed cell movement. Indeed, several chemotaxis mutants obtained by chemical mutagenesis display aberrant cGMP responses (Dicou and Brachet, 1982; Segall *et al*., 1987; Kuwayama *et al*., 1993). In one study, 10 mutants that showed no chemotactic response to either folic acid or cAMP were examined, nine of which displayed aberrant cGMP responses (Kuwayama *et al*., 1993). In particular, mutant KI8 has no detectable cGMP anymore due to the absence of all GC activity, whereas KI10 has a normal GC activity that can no longer be activated through chemoattractants. Five other mutants have a very low chemoattractant-induced cGMP response. The previously mentioned *StmF* mutants display an enhanced and prolonged cGMP response as well as a prolonged elongation in the back of cAMP waves. However, these mutants show normal chemotactic behavior when placed in wild-type generated cAMP waves, indicating that cAMP relay rather than chemotactic motility is affected in these cells (Chandrasekhar *et al*., 1995).

Several studies have indicated that cGMP exerts (part of) its action through the regulation of myosin II (reviewed in de la Roche and Cote, 2001). For example, in mutant KI10 that lacks the chemoattractant induced cGMP response, the typical phosphorylation and translocation of myosin II to the cortex in response to cAMP stimulation is lost (Liu *et al*., 1993; Liu and Newell, 1994; Dembinsky *et al*., 1996). Furthermore, in *StmF* mutants the association of myosin II to the cytoskeleton is prolonged (Ross and Newell, 1981; Liu and Newell, 1991). The identification of a myosin light chain kinase that is indirectly activated by cGMP provides further evidence for a regulatory role of cGMP on the function of myosin II (Silveira *et al*., 1998).

The identified genes define the function of cGMP

The cloning of all the main components of the cGMP signaling pathway has provided an excellent set of tools to study the effect of cGMP on chemotaxis in *Dictyostelium*. First of all, cell lines in which the two GCs or the two cGMP targets GbpC and GbpD are disrupted can unexpectedly still aggregate, albeit less robustly and slower than wild type cells (Bosgraaf *et al*., 2002a; Roelofs and Van Haastert, 2002). Detailed analysis of these mutants revealed that in a spatial gradient, chemotaxis is severely impaired and cells are less elongated. Furthermore, the cells also move slower in buffer, whereas the response to temporal chemoattractant fluctuations is essentially normal (L. Bosgraaf *et al*., unpublished data). These findings demonstrate that the production of cGMP and the presence of the cGMP targets are critical for efficient chemotaxis. The mechanism by which the cGMP pathway regulates chemotaxis was further examined by studying the phosphorylation and translocation of Myosin II in several gene knock-out mutants (Bosgraaf *et al*., 2002a). In cells that have lost both GCs or GbpC, the phosphorylation of the myosin II regulatory light chain (RLC) increases only 30% upon cAMP stimulation (wild-type 140%). In agreement with this, the *pde5*$^-$/*pde6*$^-$ cells that have a greatly enhanced and prolonged cGMP response, display an increased and especially prolonged RLC phosphorylation response (153% of wild-type). The same anomalies were found for myosin heavy chain phosphorylation, albeit less pronounced. These findings confirm the earlier hypothesis that cGMP is an important factor in the control of myosin II phosphorylation. However, it probably not the only component involved, since both *gca*$^-$/*sgc*$^-$ and *gbpc*$^-$/*gbpd*$^-$ cell lines still display a weak myosin II phosphorylation response.

Myosin II translocates to the triton insoluble cytoskeleton of the cells upon stimulation with a chemoattractant (Yumura, 1994). This translocation to the cortex peaks at 120 s and is preceded by a short release of myosin II from the triton insoluble cytoskeleton. The analysis of myosin II translocation to the cortex upon cAMP stimulation in cGMP-mutants has shed some light on the role of cGMP in this process (Bosgraaf *et al*., 2002a). The results showed that in *gca*$^-$/*sgc*$^-$ cells and in *gbpc*$^-$/*gbpd*$^-$ cells, myosin II does not translocate to the cortex anymore, but instead a prolonged and increased release of myosin from the cortex is observed. The effect of very high cGMP concentrations could be observed in the *pde5*$^-$/*pde6*$^-$ cells, which displayed very low levels of cytoskeleton-associated myosin II in unstimulated cells and an enhanced and prolonged translocation upon cAMP stimulation. These findings suggest that cGMP and its targets are essential for the translocation of myosin II to the cortex, and also indicate that a simple linear relationship between cGMP-mediated myosin II phosphorylation and -translocation to the cortex is not sufficient to explain the observations. The recent finding that three myosin heavy chain kinases localize to different parts of a moving cell further support the idea that the function of myosin II should be considered highly localized and dynamic (Liang *et al*., 2002).

Open questions and perspectives

Although we may have identified the central core in the cGMP signaling network, which allows the reinterpretation of many older observations, several findings are still not well understood. A number of cAMP-mediated responses have been suggested to be mediated by cGMP. This includes the cAMP-mediated uptake of Ca^{2+} ions,

that appears to be potentiated by cGMP (Kuwayama and Van Haastert, 1998a). Other issues in which cGMP might be involved are osmoregulation (Kuwayama and Van Haastert, 1998b), phototaxis and thermotaxis (Darcy *et al*., 1994), the induction of cAMP PDE activity (Lappano and Coukell, 1982; Van Haastert *et al*., 1982), phospolipid methylation (Alemany *et al*., 1980), and hyperpolarization (Van Duijn and Wang, 1990). With the now available mutants, it should be possible to answer these questions. Probably more difficult to solve is the understanding of the KI mutants and the mechanism of adaptation of the cGMP response.

KI mutants

The KI mutants have demonstrated that cGMP is involved in chemotaxis (Kuwayama *et al*., 1993; Kuwayama *et al*., 1995), but these mutants cannot be easily understood. KI8 lacks nearly all GC activity, does not aggregate and shows no chemotaxis to any compound. However, the *gca*/*sgc* double null mutant shows significant chemotaxis and aggregation is rather normal (Bosgraaf *et al*., 2002; Roelofs and Van Haastert, 2002). This indicates that the loss of guanylyl cyclase activity is not the only defect in these mutants, and secondly it suggests that a mutation in one gene may be responsible for the loss of activity in two guanylyl cyclases. The phenotype of KI mutants must be aggregation minus, because this was the first selection step in their isolation, followed by screening for a chemotaxis defect to folic acid and cAMP.

Adaptation of the cGMP response

The activation of GC by extracellular cAMP is very rapid (only 1 s delay) and transient due to the rapid adaptation of the enzyme ($t_{1/2}$=2.4 s) (Van Haastert, 1987). This adaptation probably involves a soluble fraction since activated GC in permeabilized cells can only be reactivated by homogenization of the cells and removal of the soluble fraction (Schoen *et al*., 1996). This soluble fraction might be a small G-protein since overexpression of constitutively active RasD enhances the desensitization of GC (Van Haastert, 1987). GC deadapts with a $t_{1/2}$ of about 90 s, but higher chemoattractant concentrations can still activate the adapted enzyme (Van Haastert, 1987). Although the mechanism of adaptation is still unknown, some indications suggest that cAMP receptors and G-proteins play an important role in this process. There exist multiple kinetic forms of the receptor with different affinities and dissociation rate constants. These receptor forms are probably cAR1 in different states of association with G-proteins (Van Haastert *et al*., 1986). It has been observed that in desensitized cells a specific transition of one receptor form to another does no longer take place; this transition was associated with the activation of the G-protein. It has been proposed that the adaptation of GC is caused by this inhibition, which is supported by the finding that the blockade is released with a similar half life as the deadaptation of GC (90 s). The use of a compound called BAL (2,3-dimercapto-1-propanol) has provided further evidence that G-proteins play a role in the adaptation of GC (Oyama, 1991; Oyama *et al*., 1991). Addition of this chemical to cells continuously activates GC, but simultaneous stimulation with cAMP returns the typical transient GC activation. Apparently, BAL can somehow activate the GC activity without activating the pathway leading to its adaptation. Interestingly, addition of cAMP and BAL to Gα2-null cells causes a continuous activation of GC, which implies that the Gα2 subunit is required for the both the activation and adaptation of GC by cAMP. However, there are several indications that Gα2 is not the only Gα subunit that plays a role in the regulation of GC. For example, Gα1 knock-out cells display a 15 s delayed cAMP induced cGMP response, whereas overexpression of Gα1 results in a decreased cGMP response with normal kinetics (Dharmawardhane *et al*., 1994). Furthermore, overexpression of Gα5, which acts as the counterpart of the folic acid receptor coupled Gα4, abolishes the folic acid induced cGMP response (Natarajan *et al*., 2000). Thus, the GC activity seems to be regulated by a complex interplay of numerous upstream proteins, including several G-alpha subunits. The exact acitvation- and adaptation mechanisms are, however, still unknown and need further investigation.

Perspectives

The identification of the genes encoding cGMP-metabolizing enzymes and target proteins will help to understand how cGMP regulates myosin phosphorylation, association of myosin filaments to the cytoskeleton and cell locomotion. Several studies are required to approach this aim. It is essential to derive reliable kinetic data on the interaction of cGMP with its target proteins GbpC and GbpD, but also on the function of the domains in these proteins. In addition, the diffusion rate and the localization of these proteins during chemotaxis need to be determined. Finally, the interaction of the cGMP-pathway with the phosphoinositol pathway may be instrumental for chemotaxis. It is likely that even with this information, chemotaxis is not easily understood, since it is a complex spatial–temporal process. In this respect mathematical models may help to get a feeling for the main regulators of chemotaxis. Previously we have made a temporal cGMP-model based on detailed kinetic experiments (Valkema and Van Haastert, 1994). In this model, the kinetics of the cGMP response are mainly determined by the different states of the receptor, the height and duration of the cGMP response are mainly determined by the PDEs, and calcium ions play a role in fine-tuning. An extension of this model encompassing the spatial properties of cGMP signaling could help in understanding the fundamentals of chemotaxis.

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